Environmental Pollution 243 (2018) 1696–1703

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

A rationale for the high limits of quantification of antibiotic resistance genes in soil☆

Gianuario Fortunato [a], Ivone Vaz-Moreira [a, b], Cristina Becerra-Castro [a, b, 1], Olga C. Nunes [b], Célia M. Manaia [a, *]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina — Laboratório Associado, Escola Superior de Biotecnologia, Rua Arquiteto Lobão Vital, 172, 4200-374, Porto, Portugal

[b] LEPABE, Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465, Porto, Portugal

ARTICLE INFO

Article history:
Received 4 May 2018
Received in revised form
25 September 2018
Accepted 26 September 2018
Available online 28 September 2018

Keywords:
Wastewater
Risk assessment
LOQ - Limit of quantification
LOD -Limit of detection
Quantitative PCR

ABSTRACT

The determination of values of abundance of antibiotic resistance genes (ARGs) *per* mass of soil is extremely useful to assess the potential impacts of relevant sources of antibiotic resistance, such as irrigation with treated wastewater or manure application. Culture-independent methods and, in particular, quantitative PCR (qPCR), have been regarded as suitable approaches for such a purpose. However, it is arguable if these methods are sensitive enough to measure ARGs abundance at levels that may represent a risk for environmental and human health. This study aimed at demonstrating the range of values of ARGs quantification that can be expected based on currently used procedures of DNA extraction and qPCR analyses. The demonstration was based on the use of soil samples spiked with known amounts of wastewater antibiotic resistant bacteria (ARB) (*Enterococcus faecalis*, *Escherichia coli*, *Acinetobacter johnsonii*, or *Pseudomonas aeruginosa*), harbouring known ARGs, and also on the calculation of expected values determined based on qPCR.

The limits of quantification (LOQ) of the ARGs (*vanA*, *qnrS*, bla_{TEM}, bla_{OXA}, bla_{IMP}, bla_{VIM}) were observed to be approximately 4 log-units *per* gram of soil dry weight, irrespective of the type of soil tested. These values were close to the theoretical LOQ values calculated based on currently used DNA extraction methods and qPCR procedures. The observed LOQ values can be considered extremely high to perform an accurate assessment of the impacts of ARGs discharges in soils. A key message is that ARGs accumulation will be noticeable only at very high doses. The assessment of the impacts of ARGs discharges in soils, of associated risks of propagation and potential transmission to humans, must take into consideration this type of evidence, and avoid the simplistic assumption that no detection corresponds to risk absence.

© 2018 Elsevier Ltd. All rights reserved.

1. Introduction

The wide dissemination of antibiotic resistant bacteria (ARB) and their genes (ARGs) as environmental contaminants, often released with treated wastewater, is considered a serious problem by the scientific community and public health authorities, for which control measures are urgently needed (Berendonk et al., 2015; Bürgmann et al., 2018; Hong et al., 2018). Water stress, due to scarcity or to deterioration of the quality of the natural freshwater resources, is a major driving force for water reuse, and inevitably, raises concerns about the associated risks of contamination of soils and of the human food chain with ARGs (Becerra-Castro et al., 2015; Negreanu et al., 2012). For these reasons, the reliable and sensitive quantification of ARGs in environmental samples exposed to human impacts is the basis of any reliable risk assessment framework.

The development of quantitative polymerase chain reaction (qPCR) remarkably improved the study of antibiotic resistance, allowing the quantification of ARGs, first in clinical samples (Espy et al., 2006), extended later on to environmental samples (Schwartz et al., 2003). The quantification of ARGs opened new perspectives to study the antibiotic resistance distribution and spread in the environment, mainly because it overcomes the

☆ This paper has been recommended for acceptance by B. Nowack.

* Corresponding author.
E-mail address: cmanaia@porto.ucp.pt (C.M. Manaia).

[1] Deceased at 28th April 2016.

https://doi.org/10.1016/j.envpol.2018.09.128
0269-7491/© 2018 Elsevier Ltd. All rights reserved.

dependence on culture-dependent methods to measure the occurrence of antibiotic resistance in a given site (Rizzo et al., 2013). In spite of its potential, qPCR has, as any other method, limitations sometimes related to the technique in itself, others due to the specificities of the matrix to be characterized. One of such limitations may be related with the sensitivity, critical to assess the impact of the discharge of contaminant ARGs in the environment, for instance in soils receiving reused water. The crucial question here is if the qPCR limits of quantification (LOQ) can be considered adequate to assess the risks associated with some ARGs (Christou et al., 2017; Manaia, 2017). The ARGs LOQ values are not expected to result only from the qPCR process. Instead they are expected to result from a combination of factors, such as the complexity of the environmental samples, the DNA extraction process, the limitation to concentrate DNA extracts, and/or the occurrence of PCR inhibitors (Combs et al., 2015; Li et al., 2017; Luby et al., 2016; Schrader et al., 2012; Sidstedt et al., 2015; Watson and Blackwell, 2000). In this study we aimed at determining the lowest range of abundance of selected ARGs and *intI1* gene that might be quantified in a soil or related matrices, using state-of-the-art procedures, namely qPCR. The possible reasons and implications of the LOQ values observed are discussed.

2. Materials and methods

In order to assess the LOQ and LOD of ARGs and related genes, soil samples were spiked with known amounts of ARB (10^7-10^2 CFU/g of soil) harboring known ARGs. Samples were collected immediately after ARB inoculation and cultivable bacteria were enumerated on different culture media, DNA was extracted and ARGs and other genes were measured based on qPCR.

2.1. Soil, ARB and ARG

Soil samples used in this study were collected as composite samples, from a greenhouse agricultural soil, located in Vila do Conde, Northern Portugal. Sampling procedures and soil and related matrices characteristics were reported before (Table S1; Becerra-Castro et al., 2017). To determine the soil dry weight, samples of soil (1 g) were weighed before (wet soil) and after drying (dry soil) by incubation at 120 °C, until no weight variation was observed, which corresponded to ~2 days.

Five ARB strains harboring ARGs or other genetic elements of interest were selected for this study (Table 1). Specifically, were analyzed: i) the *intI1* gene, encoding the class 1 integrons integrase, which is abundant in soil and considered a proxy for human impacts of antibiotic resistance (Gillings et al., 2008); ii) the genes, bla_{TEM}, bla_{OXA} *and qnrS*, which are common in wastewater habitats (Narciso-Da-Rocha et al., 2014; Szczepanowski et al., 2009); and iii) bla_{IMP}, bla_{VIM} and *vanA*, which, although reported in wastewaters, are yet not so common in the environment, being more associated with clinical samples (Vaz-Moreira et al., 2016; Yang et al., 2012).

For each strain, a calibration curve between the optical density at 610 nm (OD_{610}) and the number of colonies forming units *per* mL (CFUs/mL) was determined. Briefly, from an initial bacterial suspension with an $OD_{610} = 1$, serial dilutions with a dilution factor of 2 were prepared, for which the optical density at 610 nm and the number of CFUs *per* mL on Plate Count Agar (PCA) were determined. The curves of CFU/mL in function of the OD_{610} allowed the preparation of the bacterial suspensions with the adequate density to spike the soil slurries.

2.2. Soil slurries

Ten grams of well-homogenized soil were spiked with 3 mL of a bacterial suspension prepared in synthetic wastewater (Sousa et al., 2017) in 50 mL plastic tubes. Three different assays were settled, A1 and A2 with greenhouse soil collected in July 2014 and A3 with the same soil collected in March 2016. A1 was spiked with *Escherichia coli* strain A1FCC2; A2, spiked with *E. coli* strain A2FCC14 and *Enterococcus faecalis* strain H1EV10; and A3, spiked with *Pseudomonas aeruginosa* strain H1FC49 and *Acinetobacter johnsonii* H1PC5 (Table 1).

The bacterial suspensions were prepared from overnight cultures grown on PCA, using an OD_{610} corresponding to a bacterial density of 10^8 CFU/mL. The suspensions were then further diluted in order to reach an abundance in the initial bulk of soil of 10^7–10^2 CFU/g of wet soil. The spiked soils were thoroughly mixed and homogenized. In parallel, for each assay a soil sample supplemented with 3 mL of synthetic wastewater was used as a control (non-spiked control). Assays were prepared and analyzed in triplicate.

2.3. Quantification of ARB

For bacteria enumeration, one gram of soil was sampled from each soil slurry, suspended in 9 mL of hexametaphosphate sterile solution 1% (w/v), and serially diluted in sterile saline solution (0.85% (w/v) NaCl). Bacterial counts were made on selective culture media based on the membrane filtration method, with cellulose nitrate membranes (0.22 μm pore size, 47 mm diameter; Albet). The species *E. coli*, *A. johnsonii,* and *P. aeruginosa* were enumerated on mFC agar (fecal coliform agar, Difco BD), where they produce blue, pink or orange colonies, respectively. In addition, the species *P. aeruginosa* was also enumerated on Cetrimide agar (Pronadisa) with nalidixic acid (Sigma-Aldrich), where it selectively grows and forms green colonies. *Ent. faecalis* was enumerated on m-Enterococcus agar (Difco BD). Cultures were incubated at 30 °C for 48 h (mFC and m-Enterococcus agar) or 72 h (Cetrimide agar).

2.4. DNA extraction and quantitative PCR

Total DNA was extracted, in triplicate, from 0.25 g of soil using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc.) according to the manufacturer instructions. DNA concentration was measured with the Qubit™ fluorometer (ThermoFisher Scientific). The selected genes were quantified using the conditions described in Table S2. For the assay A1 the genes *qnrS* and bla_{TEM} were

Table 1
Bacterial strains used in this study.

Assay	Strain	Source	ARGs quantified	Reference
A1	*Escherichia coli* A1FCC2	Raw wastewater	***qnrS***; $\boldsymbol{bla_{TEM}}$	(Varela et al., 2015)
A2	*Escherichia coli* A2FCC14		$\boldsymbol{bla_{OXA}}$; $\boldsymbol{bla_{TEM}}$; ***intI1***	
	Enterococcus faecalis H1EV10	Hospital wastewater	***vanA***	
A3	*Acinetobacter johnsonii* H1PC5		$\boldsymbol{bla_{IMP}}$; $\boldsymbol{bla_{OXA}}$; ***intI1***	(Vaz-Moreira et al., 2016)
	Pseudomonas aeruginosa H1FC49		$\boldsymbol{bla_{VIM}}$; ***intI1***	
A4-A7	*Pseudomonas aeruginosa* H1FC49		$\boldsymbol{bla_{VIM}}$; ***intI1***	(Vaz-Moreira et al., 2016)

quantified; for the assay A2 the genes *intI1*, bla_{TEM}, bla_{OXA} and *vanA*; and for the assay A3 the genes *intI1*, bla_{IMP}, bla_{VIM} and bla_{OXA} (Table 1). For each assay, three independent DNA extracts were analyzed, using the Standard Curve method as described in Brankatschk et al. (2012) in a StepOne™ Real-Time PCR System (Life Technologies, Carlsbad). The quality criteria for acceptable qPCR determinations considered the possibility of interpolation to the calibration curve, the correct melting temperature of the amplicon, and absence of multiple amplification peaks or shoulders (Rocha et al., 2018). The DNA concentration to use in the quantification assays was adjusted to avoid an excess of target DNA in comparison to the primer. The lowest gene copy number obtained for each calibration curve is indicated in Table 2. The observation of amplification at Ct values below the lowest limit of the calibration curve, fitting the expected melting temperature were considered as being above the Limit of Detection (LOD), and below the LOQ (Table 2).

2.5. Influence of the soil sample mass on the observed LOQ and LOD

In an attempt to lower the copy number of genes quantifiable in the greenhouse soil slurry samples, i.e. the observed LOQ, a comparison between the PowerSoil (Mo Bio) and the FastDNA™ soil kit (MP Biomedicals) was performed. The FastDNA soil kit allows extraction from 10 g of soil, with the recommendation to resuspend in a final volume of 5 mL. The PowerSoil kit recommends the extraction from 0.25 g of soil and resuspension in a final volume of 100 μL. While the recommended proportion soil:final suspension volume is similar in both systems (1:0.5 or 1:0.4), it differs considerably in the initial amount of soil (10 g or 0.25 g), with FastDNA allowing the extraction from larger amounts of soil.

To evaluate the influence of the initial DNA extract concentration, the FastDNA soil extracts were concentrated in an attempt to lower the observed LOQ. Hence, 1 mL of FastDNA extract was concentrated using the SpeedVac concentrator (ThermoFisher Scientific) following the manufacturer instructions and re-suspended in 100 μL of ultrapure water. To compare both extraction methods and the implications of DNA concentration, namely on the PCR inhibition, the 16S rRNA gene was measured.

2.6. Effect of the type of soil or related matrix on the observed LOQ and LOD

In order to assess if the results were influenced by the type of soil or related matrix, assays were conducted with: A4) beach sand collected in the Northern region of Portugal; A5) a thermal compost produced from urban wastewater sludge currently used in gardening and agriculture; A6) commercial potting soil obtained in the retail market; and A7) a fallow soil collected in the Centre of Portugal. The physicochemical properties of these samples are shown in Table S1. Using the procedure described above, the test samples were inoculated with 10^5–10^3 CFU/g of soil/matrix of *Pseudomonas aeruginosa* strain H1FC49 (Table 1). The genes monitored to determine the observed LOQ and LOD values were the 16S rRNA, bla_{VIM} and *intI1*, as described above.

2.7. Statistical analyses

The bacterial counts and gene copy number were expressed *per* gram of soil dry weight and compared using analysis of variance (ANOVA) and the post-hoc Tukey test, with a significance level (P) of 0.05. Statistical analyses were performed using SPSS 24.0, SPSS

Table 2
Limit of quantification (LOQ) and limit of detection (LOD) values observed for the analyzed genes per gram of soil/matrix dry weight and comparison with the lowest values that could be quantified (theoretical LOQ). *LOQ – minimum quantification value (per gram dry soil) that could be interpolated in the calibration curve; **LOD – quantification values below the lowest value of the calibration curve, but with amplicons with the correct melting temperature.

Gene	Assay	Lowest value of gene copy number in the calibration curve		Observed LOQ*	Observed LOD**
		In a qPCR reaction	Correspondence *per* gram of dry weight of soil (Theoretical LOQ)§		
16S rRNA	A1	385	8.8×10^5	n.d.	n.d.
	A2		9.9×10^5		
	A3		1.0×10^6		
	A4		1.0×10^6		
	A5		1.8×10^6		
	A6		1.8×10^6		
	A7		1.0×10^6		
intI1	A2	54	1.4×10^4	n.d.	n.d.
	A3		1.4×10^4		
	A4		9.8×10^3		
	A5		1.0×10^4		
	A6		1.0×10^4	2.0×10^4	1.7×10^4
	A7		9.8×10^3	n.d.	n.d.
qnrS	A1	54	1.2×10^4	1.9×10^4	5.2×10^3
vanA	A2	54	1.4×10^4	4.7×10^4	4.4×10^3
bla_{TEM}	A1	54	1.2×10^4	1.3×10^4	5.3×10^3
	A2		1.4×10^4	6.3×10^4	8.5×10^3
bla_{OXA}	A2	64	1.5×10^4	1.4×10^5	1.2×10^4
	A3		1.7×10^4	8.3×10^4	1.1×10^4
bla_{IMP}	A3	10	2.7×10^3	1.2×10^4	8.1×10^2
bla_{VIM}	A3	22	5.9×10^3	2.3×10^4	$<6.9 \times 10^{3*}$
	A4		5.8×10^3	3.3×10^4	$<1.6 \times 10^{3*}$
	A5		1.0×10^4	3.6×10^4	$<1.6 \times 10^{3*}$
	A6		1.0×10^4	1.2×10^4	1.2×10^4
	A7		5.8×10^3	1.1×10^4	1.1×10^4

n.d., not determined; §, for the same lowest value of gene copy number in the calibration curve the correspondence *per* gram of dry weight of soil may vary, according to the humidity content of each sample. *double peaks, one with the correct melting temperature, suggest that LOD is lower than the corresponding Ct value.
Assays: A1) greenhouse soil spiked with *E. coli* strain A1FCC2; A2) greenhouse soil spiked with *E. coli* strain A2FCC14 and *Ent. faecalis* strain H1EV10; A3) greenhouse soil spiked with *P. aeruginosa* strain H1FC49 and *A. johnsonii* strain H1PC5; A4) sand spiked with *P. aeruginosa* strain H1FC49; A5) compost spiked with *P. aeruginosa* strain H1FC49; A6) potting soil spiked with *P. aeruginosa* strain H1FC49; and A7) fallow soil spiked with *P. aeruginosa* strain H1FC49.

Inc., Chicago, IL.

3. Results and discussion

ARB and ARGs are important environmental contaminants, whose growing accumulation and spread in the environment is a matter of concern (Manaia, 2017; Pruden et al., 2006; Vaz-Moreira et al., 2014). Indeed, these contaminants may not only persist in the environment but also self-replicate and proliferate. Self-replication is one, although not the unique, reason why extremely low doses of ARB or ARGs in a given environment (e.g. soil) may represent a high direct or indirect risk for human health. One possible source of soils contamination with ARB and ARGs is the water reuse in irrigation (Becerra-Castro et al., 2015; Christou et al., 2017; Negreanu et al., 2012; Wang et al., 2014). However, an overview of the literature suggests contradictory conclusions, with the observation that ARGs abundance increases in soils associated with water reuse practices being reported in parallel with no noticeable effects (Gatica and Cytryn, 2013; Negreanu et al., 2012; Wang et al., 2014). Whilst it is not the aim of this paper the discussion of possible reasons for such an apparent inconsistency, the background information gives space for a critical discussion about methodological aspects that may explain the apparently contradictory findings. This was indeed the aim of this paper: determine the abundance of ARGs that can be quantified in soils or related matrices based on commonly used DNA extraction procedures and qPCR. The final goal of studies like this is to bring a critical look about ARGs quantification in soils and stimulate the discussion, which is out of the scope of this paper, if ARGs that are not detected by this technique may, in fact, being accumulated in soil at values exceeding those considered safe levels.

3.1. Quantification of ARB

Reference bacteria were spiked in greenhouse soil at a density of 7 to 2 log-units *per* gram of wet soil as an approach to assess the LOQ and LOD that could be achieved for the different genes under analyses. Not surprisingly, the enumeration of bacteria in soil corresponded to the expected values, with a linear variation between the amount of spiked bacteria and the CFU counts in soil (Fig. 1 A–C). The only exception was a slight deviation observed for *P. aeruginosa* or *A. johnsonii* in the samples with 2 log-units inoculum, for which a higher CFU counts than expected could be due to the existence of autochthonous bacteria of these genera in soil (Fig. 1 C). Indeed, the non-spiked soil was observed to contain 2 log-units CFU/g with morphologies that, although lacking the typical morphologies considered for the analyzed strains of *P. aeruginosa* or *A. johnsonii* might, in the spiked samples, be confounded with the inoculants (data not shown).

3.2. Quantification of ARGs and other genes

In the greenhouse soil assays (A1-A3), irrespective of the amount of inoculum used, the copy number of the 16S rRNA gene was approximately 9 log-units/g of dry soil, even in non-spiked controls (Fig. 1A–C). As could be expected, the supplementation of soil with bacteria at densities at least 2 log-units lower than the original soil was not noticeable based on the qPCR quantifications. The same observation is valid for the *intI1* gene, whose presence in non-inoculated soil at densities of 6–7 log-units/g dry soil led to a narrow margin to measure exogenous inputs of this gene (Fig. 1B and C; Table 2). Although not related to the problem of the high LOQ values, the existence of a relatively high background is another drawback when the impact of exogenous ARGs sources are to be measured. This problem has been discussed before by other authors (Gatica and Cytryn, 2013).

None of the ARGs quantified in this study were detected in non-spiked greenhouse soil control samples (Fig. 1). This fact allowed the determination of the observed LOQ and LOD values for each gene, in some cases hosted by different bacterial strains. For soil slurries with inoculum size (bacterial density) $\geq$ ~3.5–4.5 log CFUs/g dry soil, the increase of the inoculum size in one log-unit corresponded to an increase of one log-unit in the gene copy number quantified for each gene (Fig. 1A–C). Such achievement is in agreement with the adequate efficiency values of the qPCR assays (Table S2). However, for soil slurries with inoculum size below ~3.5–4.5 log CFUs/g dry soil, the analyzed ARGs were below the lowest value of the calibration curve, and, therefore, could not be quantified with accuracy. Consequently, in assays A1-A3 the observed LOQ values ranged, for all analyzed ARGs 4–5 log-units *per* gram of dry soil (Fig. 1A–C, Table 2). With these assays we could, thus, conclude that, in average, to achieve a reliable qPCR quantification of a given ARG in the soil, the correspondent ARB should have at least a density of 10^4 CFUs/g dry soil. While quantification should be made based on the interpolation to a calibration curve and the LOQ value depends on the amplitude of this curve, qPCR detection may be made based on the analyses of the correct melting temperature of the amplicons. Using this rationale, the limits of detection (LOD) were determined to range 2–4 log-units of ARGs *per* gram of soil (Table 2), corresponding to ARB densities of 2–4 log CFUs/g dry soil.

3.3. Limit of quantification in different matrices

To assess the influence of the matrix in the observed LOQ and LOD values, distinct samples were tested to quantify genes harbored by *P. aeruginosa* H1FC49 (bla_{VIM} and *intI1*, Table 1).

The four matrices tested differed in physicochemical properties, mostly texture and organic matter content, in particular humic substances content (Table S1), which is known to affect the efficiency of DNA extraction (Sidstedt et al., 2015).

In this set of assays (A4-A7), the enumeration of the ARB (*P. aeruginosa*) in the different spiked samples corresponded also to the expected values (Fig. 2).

With the exception of the sand sample (Fig. 2A), with a density of 7 log-units/g dry soil for the 16S rRNA gene, the other samples presented a density of this gene of 8 log-units/g dry soil (Fig. 2B–D). The *intI1* gene was quantifiable in all samples, with the exception of the non-spiked potting soil control (Fig. 2C). The observed LOQ for the ARG bla_{VIM} was determined to be 4 log copies/g of dry soil/matrix for the different matrices tested (Fig. 2, Table 2), similar to what was observed for the greenhouse agriculture soil (Fig. 1, Table 2). These results confirm that independently of the soil matrix, the observed LOQ of ARGs is 4 log copies per gram of dry weight sample.

3.4. Influence of the sample mass and DNA concentration on the qPCR LOQ

Total DNA extractions using PowerSoil or FastDNA led to similar DNA concentrations, although different amounts (Table 3). The comparison of the 16S rRNA gene quantification in samples extracted with both methods suggest that the effect of qPCR inhibition was not observed in PowerSoil DNA extracts in contrast with the FastDNA extracts. Indeed, only after DNA dilution, the 16S rRNA gene quantification observed for the FastDNA extracts was similar to that obtained with the PowerSoil DNA extracts. It is suggested that the FastDNA kit, although allowing the recovery of a higher quantity of DNA, did not support the preparation of DNA solutions with an higher concentration than PowerSoil given the potential

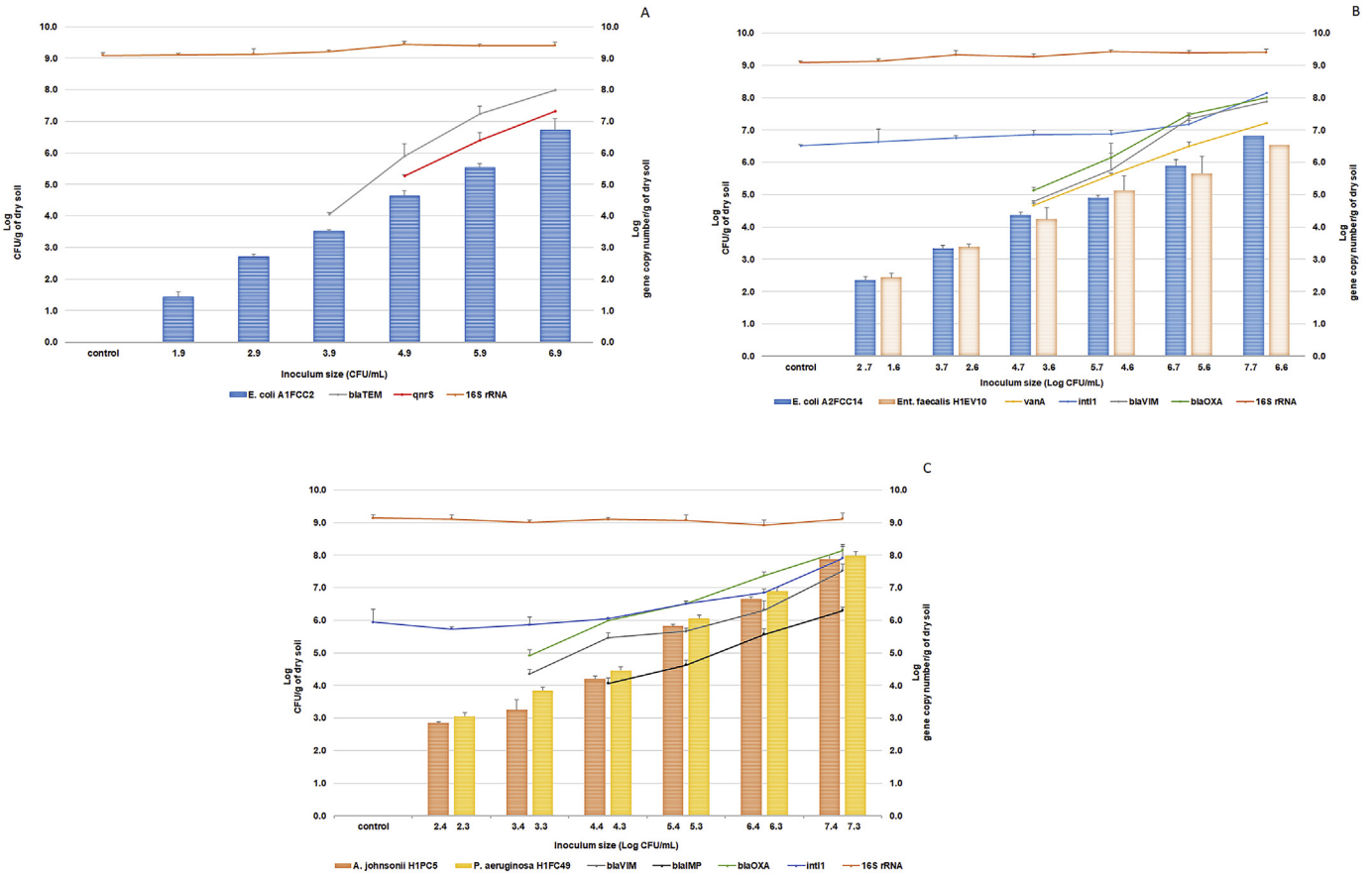

Fig. 1. Colony forming units (CFU) enumeration of spiked ARB (columns) per gram of dry weight of soil and corresponding qPCR quantification of ARG expressed in copies per gram of weight dry soil. Control refers to soil spiked with synthetic wastewater. A) spiked with *E. coli* strain A1FCC2; B) spiked with *E. coli* strain A2FCC14 and *Ent. faecalis* strain H1EV10; and C) spiked with *P. aeruginosa* strain H1FC49 and *A. johnsonii* strain H1PC5.

risks of qPCR inhibition (Table 3).

3.5. Theoretical vs experimental LOQs and implications on ARGs risk assessment

The results obtained in this study showed that the ARGs LOQ values, irrespective of the solid matrix analyzed and of the gene or of the gene host introduced in the soil, was of approximately 10^4 gene copies *per* g of dry weight of solid matrix, corresponding to an ARB density of ~10^4 CFU/g dry solid matrix, and to a ratio of approximately 10^{-3} - 10^{-5} of ARG copy number *per* total 16S rRNA gene copy number, with the exception of the sand sample where a ratio of 10^{-1} was found. An additional tenfold dilution of the bacterial inoculum did not support the quantification of any of the analyzed ARGs. The question raised was, then, if this was a practical limitation due to operational conditions or if, otherwise, it was intrinsic to the methodological constraints. Based on the average water content of a soil sample, the DNA extraction procedure and the volume of DNA extract used in the qPCR reaction, it is expected a quantification, per qPCR reaction of approximately 2–3 log-units below the real number of copies of ARGs in the sample. For example, considering the greenhouse agriculture wet soil containing approximately 20% humidity, the use of 0.25 g of wet soil for DNA extraction and of 2 μL of DNA extract in each qPCR reaction, a value of 4×10^4 copies of a ARG can be measured in a qPCR reaction when analyzing a soil sample containing 10^7 gene copies *per* gram of wet soil (Table 4, bold numbers). Therefore, from the real abundance of an ARG in the soil to what can be measured there is a reduction by a factor of 4×10^{-3}, only due to DNA extraction and qPCR reaction. This means that in a soil containing one thousand ARG copies *per* gram of soil wet weight, which can be meaningful in terms of human health, one would expect to be able to amplify 4 ARG copies by PCR (Table 4), which is not a realistic expectation with the most commonly used real-time PCR protocols. Using qPCR procedures, like those of the present study, where the lowest value of gene copy number in the calibration curve was around 20–60, the theoretical and observed LOQ values were shown to be in agreement for most of the ARGs and all the matrices analyzed (Table 2). These results suggest that methodological improvements are required to achieve lower LOQ values. The extraction of DNA from a larger soil mass could be a positive contribution. However, the extraction and concentration of qPCR inhibitors is a problem to overcome, as discussed above (Table 3). Another helpful modification may be the use of a larger volume of DNA extract per qPCR reaction, which can increase the LOQ in about 1 log-unit. However, this procedure has the potential to increase inhibition, either due to the excess of DNA that can inhibit PCR or due to the increasing amount of inhibitors in the extract. Droplet digital PCR (ddPCR) has been proposed as a good alternative to overcome limitations of qPCR. In average, ddPCR can reduce in about 10 times the LOQ of ARGs in comparison to qPCR (Cavé et al., 2016). Nevertheless, a major constraint that results from DNA extraction from soil and the avoidance of PCR inhibitors seems to represent a bottleneck still to solve.

The discussion about the risks of contamination of the human food chain with ARB and ARGs due to intended or inadvertent

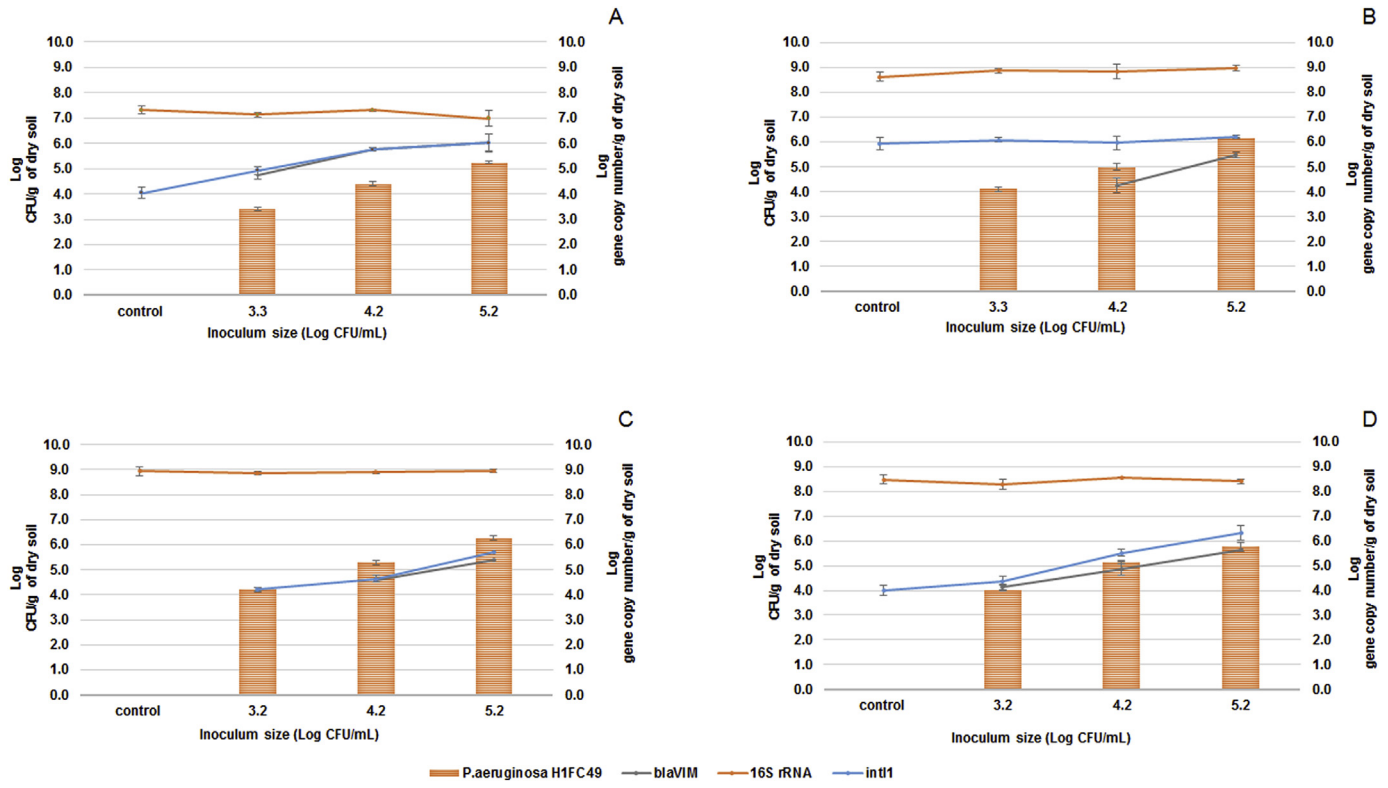

Fig. 2. Colony forming units (CFU) enumeration of spiked ARB (columns) per gram of dry weight of soil and corresponding qPCR quantification of ARG expressed in copies per gram of weight dry soil/matrix, for A) sand; B) compost; C) potting soil; and D) fallow soil.

Table 3
Comparison of 16S rRNA gene quantification in DNA extracts (E) obtained with FastDNA™ soil kit (MP Biomedicals) or PowerSoil kit (Mo Bio).

DNA extraction method	Amount of soil and extract volume	DNA extract	DNA concentration (μg/mL)	Log (gene copy number/g soil dry weight)
FastDNA™ soil kit (MP biomedicals)	5 g soil	E	11.8	7.4 ± 0.20^{a}
	5 mL extract (E)	10 C	110.7	7.8 ± 0.05^{b}
	10 × concentrated (10 C)	10 C + 10 D	11.1	8.6 ± 0.03^{c}
PowerSoil (Mo Bio)	0.25 g soil	E	16.5	8.8 ± 0.07^{c}
	100 μL extract (E)	10 D	1.7	8.8 ± 0.13^{c}
	10 × diluted (10 D)			

E, DNA extract; 10 C, DNA extract concentrated 10×; 10 D, DNA extract diluted 10×.
a,b,c - statistically different ($p < 0.05$) using ANOVA.

Table 4
Estimation of the gene copy number in a qPCR reaction using DNA extracts obtained from 0.25 g of wet soils with different densities of a given gene. It is assumed that i) the final DNA extracts are suspended in 100 μL; ii) DNA extraction has an efficiency of 100%; iii) 20% of soil mass is water; iv) each qPCR reaction uses 2 μL of DNA solution.

Gene copies *per* gram of wet soil	Mass of wet soil used (g)	Humidity percentage (%)	Volume of DNA extract (μL)	Volume of DNA used in the qPCR (μL)	Gene copy number expected to be quantified in a qPCR reaction
$\mathbf{1 \times 10^{7}}$					$\mathbf{4 \times 10^{4}}$
1×10^{6}					4×10^{3}
1×10^{5}	0.25	20	100	2	4×10^{2}
1×10^{4}					4×10^{1}
1×10^{3}					4×10^{0}

water reuse has been intensified over the last years. In this discussion, one of the central issues is the technical capability to detect and quantify ARB and ARGs that may accumulate or proliferate in soil, representing a threat to humans and animals. It is recognized that it is very difficult to propose a threshold for the maximum admissible emission values of ARB and ARGs, for instance by wastewater treatment plants. However, it is consensual that extremely low doses of some ARB, namely those harboring last generation ARGs such as those encoding carbapenem or colistin resistance, may represent high risks for humans and the environment. The ranking of risks and the concerns raised by the presence of specific ARGs cannot be made out of a context. It is important to take into consideration the soil history and properties as well as the land use and end-users. In this way, when such evaluations are to be made, it is important to consider that qPCR, the current methodology used to quantify ARGs, may fail to quantify potentially

hazardous biological contaminants, whose abundance is below 10^4 CFU *per* g of soil wet weight.

4. Conclusions

The key message of this article is clear – the results of ARGs quantification in soils using state-of-the-art procedures need to be interpreted with caution. We demonstrated that with commonly used qPCR procedures it is not possible to quantify ARGs in solid matrices, independently of the type, where they are at or below an abundance of one thousand copies *per* gram of soil dry weight. Moreover, we demonstrated that these results were not due to practical biases, as theoretical and observed LOQ values were in the same range. Values below the LOQ may represent an overwhelming amount of ARB and ARGs, mainly if one considers the high ecological fitness of bacteria, and high capability to reproduce in the environment.

Disclaimer

The content of this publication reflects only the authors' views and the Research Executive Agency is not responsible for any use that may be made of the information it contains.

Acknowledgments

This work is part of a project that has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 675530.

This work was supported by National Funds from FCT – Fundação para a Ciência e a Tecnologia, Portugal, through projects UID/Multi/50016/2013, WaterJPI/0001/2013 STARE – "Stopping Antibiotic Resistance Evolution", by project POCI-01-0145-FEDER-006939 (LEPABE– UID/EQU/00511/2013), funded by European Regional Development Fund through COMPETE2020 – Programa Operacional Competitividade e Internacionalização (POCI), and by national funds through FCT – Fundação para a Ciência e a Tecnologia; project "LEPABE-2-ECO-INNOVATION" – NORTE-01-0145-FEDER-000005, funded by Norte Portugal Regional Operational Programme (NORTE 2020), under PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund.

IVM and CBC were supported by the FCT grants SFRH/BPD/87360/2012 and SFRH/BPD/87152/2012, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2018.09.128.

References

Becerra-Castro, C., Lopes, A.R., Teixeira, S., Silva, M.E.F., Pimenta, E., Manaia, C.M., Nunes, O.C., 2017. Characterization of bacterial communities from Masseiras, a unique Portuguese greenhouse agricultural system. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 110, 665–676. https://doi.org/10.1007/s10482-017-0833-7.

Becerra-Castro, C., Lopes, A.R., Vaz-Moreira, I., Silva, E.F., Manaia, C.M., Nunes, O.C., 2015. Wastewater reuse in irrigation: a microbiological perspective on implications in soil fertility and human and environmental health. Environ. Int. 75. https://doi.org/10.1016/j.envint.2014.11.001.

Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F., Martinez, J.L., 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 13, 310–317. https://doi.org/10.1038/nrmicro3439.

Brankatschk, R., Bodenhausen, N., Zeyer, J., Burgmann, H., 2012. Simple absolute quantification method correcting for quantitative PCR efficiency variations for microbial community samples. Appl. Environ. Microbiol. 78, 4481–4489. https://doi.org/10.1128/AEM.07878-11.

Bürgmann, H., Frigon, D., Gaze, W.H., Manaia, C.M., Pruden, A., Singer, A.C., Smets, B.F., Zhang, T., 2018. Water and sanitation: an essential battlefront in the war on antimicrobial resistance. FEMS Microbiol. Ecol. https://doi.org/10.1093/femsec/fiy101.

Cavé, L., Brothier, E., Abrouk, D., Bouda, P.S., Hien, E., Nazaret, S., 2016. Efficiency and sensitivity of the digital droplet PCR for the quantification of antibiotic resistance genes in soils and organic residues. Appl. Microbiol. Biotechnol. 100, 10597–10608. https://doi.org/10.1007/s00253-016-7950-5.

Christou, A., Agüera, A., Bayona, J.M., Cytryn, E., Fotopoulos, V., Lambropoulou, D., Manaia, C.M., Michael, C., Revitt, M., Schröder, P., Fatta-Kassinos, D., 2017. The potential implications of reclaimed wastewater reuse for irrigation on the agricultural environment: the knowns and unknowns of the fate of antibiotics and antibiotic resistant bacteria and resistance genes – a review. Water Res. 123, 448–667. https://doi.org/10.1016/j.watres.2017.07.004.

Combs, L.G., Warren, J.E., Huynh, V., Castaneda, J., Golden, T.D., Roby, R.K., 2015. The effects of metal ion PCR inhibitors on results obtained with the Quantifiler® Human DNA Quantification Kit. Forensic Sci. Int. Genet. 19, 180–189. https://doi.org/10.1016/j.fsigen.2015.06.013.

Espy, M.J., Uhl, J.R., Sloan, L.M., Buckwalter, S.P., Jones, M.F., Vetter, E.A., Yao, J.D.C., Wengenack, N.L., Rosenblatt, J.E., Cockerill, F.R., Smith, T.F., 2006. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin. Microbiol. Rev. 19, 165–256. https://doi.org/10.1128/CMR.19.1.165-256.2006.

Gatica, J., Cytryn, E., 2013. Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. Environ. Sci. Pollut. Res. Int. 20, 3529–3538. https://doi.org/10.1007/s11356-013-1505-4.

Gillings, M., Boucher, Y., Labbate, M., Holmes, A., Krishnan, S., Holley, M., Stokes, H.W., 2008. The evolution of class 1 integrons and the rise of antibiotic resistance. J. Bacteriol. 190, 5095–5100. https://doi.org/10.1128/JB.00152-08.

Hong, P.Y., Julian, T.R., Pype, M.L., Jiang, S.C., Nelson, K.L., Graham, D., Pruden, A., Manaia, C.M., 2018. Reusing treated wastewater: consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes. Water 10 (3). Switzerland. https://doi.org/10.3390/w10030244.

Li, A.-D., Metch, J.W., Wang, Y., Garner, E., Zhang, A.N., Riquelme, M.V., Vikesland, P.J., Pruden, A., Zhang, T., 2017. Effects of sample preservation and DNA extraction on enumeration of antibiotic resistance genes in wastewater. FEMS Microbiol. Ecol. https://doi.org/10.1093/femsec/fix189.

Luby, E., Ibekwe, A.M., Zilles, J., Pruden, A., 2016. Molecular methods for assessment of antibiotic resistance in agricultural ecosystems: prospects and challenges. J. Environ. Qual. 45, 441–453. https://doi.org/10.2134/jeq2015.07.0367.

Manaia, C.M., 2017. Assessing the risk of antibiotic resistance transmission from the environment to humans: non-direct proportionality between abundance and risk. Trends Microbiol. 25 (3), 173–181. https://doi.org/10.1016/j.tim.2016.11.014.

Narciso-Da-Rocha, C., Varela, A.R., Schwartz, T., Nunes, O.C., Manaia, C.M., 2014. BlaTEM and vanA as indicator genes of antibiotic resistance contamination in a hospital-urban wastewater treatment plant system. J. Glob. Antimicrob. Resist. 2, 309–315. https://doi.org/10.1016/j.jgar.2014.10.001.

Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E., 2012. Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. Environ. Sci. Technol. 46, 4800–4808. https://doi.org/Doi 10.1021/Es204665b.

Pruden, A., Pei, R., Storteboom, H., Carlson, K.H., 2006. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ. Sci. Technol. 40, 7445–7450.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., K.D, F., 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci. Total Environ. 447, 345–360. https://doi.org/10.1016/j.scitotenv.2013.01.032.

Rocha, J., Cacace, D., Kampouris, I., Guilloteau, H., Jäger, T., Marano, R.B.M., Karaolia, P., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., 2018. Inter-laboratory calibration of quantitative analyses of antibiotic resistance genes. J. Environ. Chem. Eng.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. 113 (5), 1014–1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U., 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43, 325–335. https://doi.org/FEM325 [pii] 10.1111/j.1574-6941.2003.tb01073.x.

Sidstedt, M., Jansson, L., Nilsson, E., Noppa, L., Forsman, M., Rådström, P., Hedman, J., 2015. Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. Anal. Biochem. 487, 30–37. https://doi.org/10.1016/j.ab.2015.07.002.

Sousa, J.M., Macedo, G., Pedrosa, M., Becerra-Castro, C., Castro-Silva, S., Pereira, M.F.R., Silva, A.M.T., Nunes, O.C., Manaia, C.M., 2017. Ozonation and UV254nm radiation for the removal of microorganisms and antibiotic resistance genes from urban wastewater. J. Hazard Mater. 323, 434–441. https://doi.org/10.1016/j.jhazmat.2016.03.096.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.H., Gutzkow, T., Eichler, W., Puhler, A., Schluter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology (N. Y.) 155, 2306–2319. https://doi.org/mic.0.028233-0 [pii]10.1099/mic.0.028233-0.

Varela, A.R., Macedo, G.N., Nunes, O.C., Manaia, C.M., 2015. Genetic characterization

of fluoroquinolone resistant Escherichia coli from urban streams and municipal and hospital effluents. FEMS Microbiol. Ecol. 91. https://doi.org/10.1093/femsec/fiv015.

Vaz-Moreira, I., Nunes, O.C., Manaia, C.M., 2014. Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome. FEMS Microbiol. Rev. 38 (4), 761–778. https://doi.org/10.1111/1574-6976.12062.

Vaz-Moreira, I., Varela, A.R., Pereira, T.V., Fochat, R.C., Manaia, C.M., 2016. Multidrug resistance in quinolone-resistant gram-negative bacteria isolated from hospital effluent and the municipal wastewater treatment plant. Microb. Drug Resist. 22. https://doi.org/10.1089/mdr.2015.0118.

Wang, F.H., Qiao, M., Lv, Z.E., Guo, G.X., Jia, Y., Su, Y.H., Zhu, Y.G., 2014. Impact of reclaimed water irrigation on antibiotic resistance in public parks, Beijing, China. Environ. Pollut. 184, 247–253. https://doi.org/10.1016/j.envpol.2013.08.038.

Watson, R.J., Blackwell, B., 2000. Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can. J. Microbiol. 46, 633–642. https://doi.org/10.1139/w00-043.

Yang, Y., Zhang, T., Zhang, X.X., Liang, D.W., Zhang, M., Gao, D.W., Zhu, H.G., Huang, Q.G., Fang, H.H.P., 2012. Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. Appl. Microbiol. Biotechnol. 95, 1351–1358. https://doi.org/10.1007/s00253-011-3810-5.